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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant :	Charles D. Jones	
Serial No.:	331,042	Group Art Unit: 121
Filed :	December 16, 1981	Examiner: R. Schwartz
For :	ANTIESTROGENIC AND ANTI-ANDROGENIC BENZOTHIOPHENE	
Docket No.:	X-5526A	

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DECLARATION UNDER 37 C.F.R. 1.132

Michael L. Hanlin declares as follows:

I am responsible for the screening and testing of potential antiandrogenic agents, and for developing and carrying out studies of androgen metabolism in male accessory sex organs. I am employed in the central nervous system-endocrinology research division of Eli Lilly and Company, and have been so employed since 1966. I obtained my Bachelor of Science degree in 1965 at Butler University, Indianapolis, Indiana with a dual major in zoology and chemistry. I earned my Master of Science degree in 1970 at Indiana University in pharmacology. I was made responsible for the anti-androgen area of Lilly's physiological research in 1968, and have published about four papers in the field of androgen research.

Experiments have been carried out by me or by others under my direct supervision in which compounds pertinent to the above-named patent application were tested. The compounds discussed here are (I) 6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-piperidinoethoxy)benzoyl]benzo[b]thiophene, and (II) 6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-pyrrolidinoethoxy)benzoyl]benzo[b]thiophene. The compounds will be referred to in this document as I and II for convenience.

A. Test to determine in vitro competition for androgen binding

The artificial androgen, methyltrienolone-17α-methyl-^{3}H was used, because it has equal or greater affinity for cytoplasmic

receptors than does tritiated dihydrotestosterone, but the trienolone (usually called ^{3}H-R1881) exhibits less nonspecific binding to non-receptor proteins in cytoplasm. The animals used were immature male rats which had been castrated by the scrotal route 24 hours before sacrifice. Ventral prostate tissue was pooled and homogenized in three volumes (weight/volume) of 10mM tris-(hydroxymethyl)aminomethane, hydrochloride buffer at pH 8.0, containing 1mM EDTA, 0.25M of sucrose and 0.01M sodium molybdate (buffer A). The homogenate was centrifuged at 100,000 g for 1 hour at 4°C. and the supernatant was used to measure cytoplasmic ^{3}H-R1881 binding.

Triplicate aliquots of 50 µl each of cytoplasmic supernates were incubated with 5.0 nM ^{3}H-R1881 in buffer B (the same as buffer A described above except that it contains 10 percent glycerol and no molybdate) in a total volume of 0.5 ml. Parallel incubations containing various concentrations of unlabeled ^{3}H-R1881 and the test compounds were also assayed. The samples were incubated for 4 hours at 4°C. with gentle mixing every 30 minutes, and the incubations were terminated by the addition of 0.5 ml. of an ice cold suspension of 1 percent activated charcoal and 0.1 percent dextran in buffer A without sucrose. Ten minutes after adding the charcoal suspension, the samples were centrifuged at 1000 g for 15 minutes at 4°C., and aliquots of 0.5 ml. were obtained for scintillation counting. The results are shown in the table below.

Table I

	Molar Concentration of R1881 or Compound I				
Compound	0	10^{-9}	10^{-8}	10^{-7}	10^{-6}
R1881	8634*	6565	5307	4821	4810[a]
Percent Inh.	--	54	87	99	100
Compound I	8634	8959	8838	8796	9040
Percent Change	--	+8	+5	+4	+11

*Values represent DPM's bound in the assay. Triplicate determinations. 5.0×10^{-9}M was the concentration of ^{3}H-R1881 in each incubation tube. Total cytoplasmic binding of ^{3}H-R1881 with no unlabeled compound present = 8634 ± 159 DPM's.

[a] 5×10^{-6}M unlabeled R1881 = maximal displacement = 100 percent inhibition of ^{3}H-R1881 binding.

	Molar Concentration of R1881 or Compound II				
Compound	0	10^{-9}	10^{-8}	10^{-7}	10^{-6}
R1881	6062*	5674	4400	3856[b]	3884
Percent Inh.	--	18	75	100	99
Compound II	6062	6986	6956	6998	6949
Percent Change	--	+41	+40	+42	+40

*Values represent DPM's bound in the assay. Duplicate determinations. 5.0×10^{-9}M was the concentration of ^{3}H-R1881 in each incubation tube. Total cytoplasmic binding of ^{3}H-R1881 with no unlabeled compound present = 6062 DPM's.

[b] 5×10^{-7}M unlabeled R1881 = maximal displacement = 100 percent inhibition of ^{3}H-R1881 binding.

The results of this experiment show that neither compound has any substantial affinity for binding to prostatic androgen receptors. The increased total binding observed for compound II is probably the result of increased non-specific binding in this assay.

B. In vitro determination of androgen receptor concentration following in vivo treatment with test compounds

Adult male rats were castrated 48 hours prior to sacrifice. A single injection of the test compound was administered 1, 17 or 24 hours before sacrifice. Ventral prostates were removed, weighed and individually prepared for androgen receptor determination. The results are shown in Table II.

Table II

THE EFFECT OF A SINGLE INJECTION OF COMPOUND I UPON THE BINDING OF ^{3}H-R1881 IN THE CASTRATE ADULT MALE RAT

Treatment Group		Final Body Wt. (gm ± S.E.)	VP Wt. (mg ± S.E.)	^{3}H-R1881 Bound Fm/mg Cyto Protein
Vehicle Control[a]	(4)	289 ± 8	362 ± 21	35 ± 4
Compound I[b]	(4)	294 ± 5	341 ± 19	33 ± 2
Compound I[c]	(4)	284 ± 13	353 ± 24	42 ± 12

( ) = number of rats/group.

[a]0.1 ml corn oil s.c. 17 hrs. prior to sacrifice.
[b]0.35 mg/kg s.c. 17 hrs. prior to sacrifice.
[c]0.35 mg/kg s.c. 1 hr. prior to sacrifice.

THE EFFECT OF A SINGLE INJECTION OF COMPOUND II UPON THE BINDING OF ^{3}H-R1881 IN THE CASTRATE ADULT MALE RAT

Treatment Group		Av. VP Wt.[f] (mg)	^{3}H-R1881 Bound Fm/mg Cyto Protein
Vehicle Control[d]	(3)	177	24
Compound II[e]	(4)	170	21

( ) = number of rats/group.

[d]0.1 ml. corn oil s.c. 24 hrs. prior to sacrifice.
[e]1.0 mg/kg s.c. 24 hrs. prior to sacrifice.
[f]Ventral prostrates of each group were pooled and binding determined on the pool in this experiment.

Adult male rats were castrated and maintained untreated for 5 days to allow metabolism of endogenous androgens. Experimental groups were then treated once daily for 7 consecutive days with test compound, estradiol, or estradiol combined with the test

compound. The rats were sacrificed 24 hours after the last treatment, and ventral prostates were removed, weighed and pooled for androgen receptor assay. The results are shown in Table III.

Table III

THE EFFECT OF CHRONIC ADMINISTRATION OF ESTRADIOL OR COMPOUND I SEPARATELY OR IN COMBINATION UPON VENTRAL PROSTATE (VP) WEIGHT AND BINDING OF ^{3}H-R1881 IN THE CASTRATE ADULT MALE RAT

Treatment Group		Final Body Wt. (gm ± S.E.)	^{3}H-R1881 Fm/mg Cyto Protein	VP Wt. (mg ± S.E.)
Vehicle Control[a]	(7)	298 ± 14	63	30 ± 4
E^2 [b]	(7)	291 ± 8	269	32 ± 5
Compound I[c]	(7)	282 ± 12	77	31 ± 4
Compound I[d]	(7)	293 ± 3	41	30 ± 2
E^2 + Compound I[e]	(7)	293 ± 6	85	28 ± 2
E^2 + Compound I[f]	(7)	288 ± 4	84	28 ± 2

( ) = number of rats/group.

[a]0.1 ml. corn oil/day s.c.
[b]Estradiol 5 mg/kg/day s.c.
[c]5.0 mg/kg/day s.c.
[d]0.5 mg/kg/day s.c.
[e]Estradiol + Compound I 5.0 mg/kg/day s.c.
[f]Estradiol + Compound I 0.5 mg/kg/day s.c.

THE EFFECT OF CHRONIC ADMINISTRATION OF ESTRADIOL OR COMPOUND II[g] SEPARATELY OR IN COMBINATION UPON VENTRAL PROSTATE (VP) WEIGHT AND BINDING OF ^{3}H-R1881 IN THE CASTRATE ADULT MALE RAT

Treatment Group		Final Body Wt. (gm ± S.E.)	^{3}H-R1881 Fm/mg Cyto Protein	VP Wt. (mg ± S.E.)
Vehicle Control[a]	(8)	311 ± 2	12	28 ± 3
E^2 [b]	(8)	298 ± 3	40	32 ± 2
Compound II[h]	(7)	278 ± 5	31	35 ± 1
E^2 + Compound II[i]	(6)	278 ± 4	25	38 ± 12

( ) = number of rats/group.

[g]Treatment was for 8 consecutive days.
[a]0.1 ml. corn oil/day s.c.
[b]Estradiol 5 µg/kg/day s.c.
[h]5 mg/kg/day s.c.
[i]Combination of E^2 and Compound II.

The results of the above experiments show that when estradiol and compound I were administered simultaneously, there was a blockade of the increase in receptor concentration observed with estradiol alone, indicating that compound I blocks the androgen receptor inducing effect of estradiol. The effect did not occur in the tests with compound II.

C. Castrate rat anti-androgen assays

Immature male rats were castrated and held untreated for 3 days to allow metabolism of endogenous androgens. On the fourth day after castration, the rats were treated with exogenous androgen and the test compound once each day for 7 consecutive days. Twenty-four hours after the last treatment the rats were sacrificed, and seminal vesicles and ventral prostates were removed and weighed. The results are shown in the following Table IV.

Table IV

THE EFFECT OF SUBCUTANEOUS ADMINISTRATION OF COMPOUNDS I AND II UPON ANDROGEN STIMULATION OF SEMINAL VESICLE (SV) AND VENTRAL PROSTATE (VP) WEIGHT GAIN IN THE CASTRATE IMMATURE MALE RAT

Treatment		Final Body Wt. (gm ± S.E.)	S.V. Wt. (mg ± S.E.)	V.P. Wt. (mg ± S.E.)
T.P. Control[a]	(16)	116 ± 5	39.6 ± 2.2	43.5 ± 2.9
Vehicle Control[b]	(10)	119 ± 6	11.4 ± 0.6	11.4 ± 0.4
T.P. + Compound I[c]	(9)	111 ± 6	33.3 ± 2.9	29.0 ± 3.6
T.P. + Compound I[d]	(10)	111 ± 7	32.3 ± 2.3	31.1 ± 2.2
T.P. + Compound I[e]	(10)	114 ± 7	28.8 ± 1.0	34.0 ± 1.9

( ) = number of rats/group.

[a] Testosterone propionate 0.20 mg/kg/day s.c.
[b] 0.1 ml. corn oil/day s.c.
[c] 5.0 mg/kg/day s.c.
[d] 0.5 mg/kg/day s.c.
[e] 0.05 mg/kg/day s.c.

Table IV (cont)

Treatment		Final Body Wt. (gm ± S.E.)	S.V. Wt. (mg ± S.E.)	V.P. Wt. (mg ± S.E.)
T.P. Control[a]	(12)	116 ± 3	54.0 ± 2.3	48.0 ± 2.4
Vehicle Control[b]	(7)	123 ± 3	10.2 ± 0.6	8.0 ± 0.5
T.P. + Compound I[f]	(7)	118 ± 4	19.9 ± 2.0	17.3 ± 2.6

( ) = number of rats/group.

[a]Testosterone propionate 0.20 mg/kg/day s.c.
[b]0.1 ml. corn oil/day s.c.
[f]0.05 mg/kg/day s.c.

Treatment		Final Body Wt. (gm ± S.E.)	S.V. Wt. (mg ± S.E.)	V.P. Wt. (mg ± S.E.)
T.P. Control[g]	(8)	115 ± 4	51.0 ± 2.6	46.2 ± 1.9
Vehicle Control[b]	(6)	116 ± 2	11.8 ± 1.1	8.9 ± 1.2
T.P. + Compound I[h]	(5)	116 ± 4	28.9 ± 1.7	26.3 ± 1.9
T.P. + Compound I[i]	(5)	114 ± 1	29.4 ± 1.6	30.8 ± 3.6
T.P. + Compound I[j]	(5)	120 ± 3	27.0 ± 1.1	25.6 ± 2.9

( ) = number of rats/group.

[g]Testosterone propionate 0.25 mg/kg/day s.c.
[b]0.1 ml. corn oil/day s.c.
[h]2.5 mg/kg/day.
[i]0.5 mg/kg/day.
[j]0.1 mg/kg/day.

Treatment		Final Body Wt. (gm ± S.E.)	S.V. Wt. (mg ± S.E.)	V.P. Wt. (mg ± S.E.)
T.P. Control[a]	(9)	120 ± 2	68.2 ± 2.6	66.0 ± 2.5
Vehicle Control[b]	(5)	114 ± 3	11.5 ± 0.8	9.8 ± 0.9
T.P. + Compound II[k]	(5)	112 ± 3	74.3 ± 3.4	66.4 ± 1.9
T.P. + Compound II[l]	(5)	113 ± 3	66.3 ± 6.0	67.0 ± 5.3
T.P. + Compound II[m]	(5)	113 ± 2	70.5 ± 7.2	67.0 ± 5.9

( ) = number of rats/group.

[a]Testosterone propionate 0.20 mg/kg/day s.c.
[b]0.1 ml corn oil/day s.c.
[k]5.0 mg/kg/day s.c.
[l]1.0 mg/kg/day s.c.
[m]0.2 mg/kg/day s.c.

The results of the above-reported experiments show that compound I inhibits weight gain of the seminal vesicles and ventral prostates, whereas the treatments with compound II and testosterone propionate produced weight gains of those organs approximately equal to treatment with testosterone propionate alone.

Differences between compounds I and II were observed in the androgen receptor concentration following in vivo treatment, and in in vivo anti-androgen activity. In Table III, it should be noted that compound I blocked the increase in androgen receptor concentration following estradiol treatment, whereas compound II did not substantially block the increase and appeared to contribute some increase of its own. In Table IV, compound II showed no inhibition of androgen stimulation of accessory organ weights, but compound I markedly decreased androgen stimulation in all replicates.

I conclude from my tests that compound I is a potent anti-androgen, but that compound II appears to be devoid of anti-androgenic activity.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Michael L. Hanlin

October 26, 1982
Date